Expression and characterization of the *Trypanosoma cruzi* dihydrofolate reductase domain

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Abstract

We have cloned and expressed in *Escherichia coli* a 702-base pair gene coding for the dihydrofolate reductase (DHFR) domain of the bifunctional dihydrofolate reductase-thymidylate synthase (DHFR-TS) from *Trypanosoma cruzi*. The DHFR domain was purified to homogeneity by methotrexate-Sepharose chromatography followed by an anion-exchange chromatography step in a mono Q column, and displayed a single 27-kDa band on SDS-PAGE. Gel filtration showed that the catalytic domain was expressed as a monomer. Kinetic parameters were similar to those reported for the wild-type bifunctional enzyme with K_m values of 0.75 μM for dihydrofolate and 16 μM for NADPH and a k_{cat} value of 16.5 s^{-1}. *T. cruzi* DHFR is poorly inhibited by trimethoprim and pyrimethamine and the inhibition constants were always lower for the bifunctional enzyme. The binding of methotrexate was characteristic of a class of inhibitors that form an initial complex which isomerizes slowly to a tighter complex and are referred to as 'slow, tight-binding' inhibitors. While the slow-binding step of inhibition was apparently unaffected in the individually expressed DHFR domain, the overall inhibition constant was two-fold higher as a consequence of the superior inhibition constant value obtained for the initial inhibitory complex.

Keywords: *Trypanosoma cruzi*; Dihydrofolate reductase; Protozoal enzymes; Heterologous expression; Folate metabolism

1. Introduction

Thymidylate synthase (TS) and dihydrofolate reductase (DHFR) catalyze sequential reactions in the biosynthesis of dTMP. TS catalyzes the conversion of dUMP and 5,10-methylenetetrahydrofolate to dTMP and dihydrofolate (H_2folate),

Abbreviations: DHFR-TS, dihydrofolate reductase-thymidylate synthase; Pyr, pyrimethamine; TMP, trimethoprim; MTX, methotrexate; H_2folate, dihydrofolate; PCR, polymerase chain reaction.

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while DHFR is responsible for the subsequent step of regeneration of tetrahydrofolate from H_2folate which is necessary for the synthesis of dTMP. In most organisms, DHFR and TS exist as separate monofunctional enzymes [1,2]. TS is usually a homodimer protein of 60–70 kDa while DHFR is an 18–25 kDa monomer. In contrast, in protozoa, both enzymes are on the same polypeptide chain, with the DHFR domain at the amino terminus and the TS domain at the carboxy terminus. The native bifunctional enzyme is a dimer of two of such subunits with a molecular weight ranging between 110 and 140 kDa [3–5]. There is evidence indicating functional interactions between the individual domains of the DHFR-TS dimer, presumably communicated via protein conformational changes. For example, binding of MTX to one DHFR domain prevents binding to the other and substrate channeling of H_2folate from the active site of TS to that of DHFR has been demonstrated [6]. An X-ray structure for the *Leishmania major* bifunctional protein showed a highly positive electrostatic potential surface around and between both folate binding sites that could explain this phenomenon in terms of electrostatic channeling [7].

TS and DHFR are targets for chemotherapy, as inhibition of either enzyme disrupts the dTMP cycle and results in the death of cells. DHFR has been successfully used as a drug target since it has diverged greatly through evolution and DHFRs from different sources can show dramatic differences in their inhibition by folate analogues. The most extensively studied and successful DHFR inhibitors are analogues of 2,4-diaminopyrimidines, which include trimethoprim (TMP) and pyrimethamine (Pyr), and analogues of 2,4-diaminopteridine such as methotrexate (MTX). Both TMP and Pyr are paradigms of selective enzyme inhibitors and, alone or in combination with sulfa drugs, have been employed in the treatment of diseases caused by protozoa, such as malaria [8], toxoplasmosis [9] and *Pneumocystis carinii* pneumonia [10]. However, the classical antifolates have not been clinically useful in the treatment of diseases caused by other parasitic protozoa.

In contrast, MTX is an extremely potent inhibitor of DHFR from all sources. It is used in the treatment of neoplasia but shows marginal selective toxicity. Binding kinetics of MTX with its target have been studied with DHFRs from several sources [11,12]. These studies indicated that the complexes initially formed undergo some kind of conformational change, the effect of which is to increase the tightness of binding.

Since the chemotherapy of Chagas' disease, of which the etiological agent is *Trypanosoma cruzi*, remains an unsolved problem, the identification and exploitation of the clear differences between DHFRs from the parasite and its mammalian host should allow for the development of effective drugs. We have previously performed the purification and characterization of DHFR-TS from *T. cruzi* [13] and now we report the heterologous expression, purification and characterization of the individual DHFR domain. With a smaller protein it will presumably be easier to perform a structural analysis and to apply techniques like nuclear magnetic resonance since the bifuctional homodimeric enzyme is considered too large for such detailed studies. Likewise, the availability of the domain will allow a better understanding of the structure and function of the bifunctional enzyme which might contribute towards a rational design of specifically targeted drugs.

2. Materials and methods

2.1. Materials

All reagents used were purchased from Sigma and of the highest purity available. Dihydrofolate was prepared from folic acid by the method of Blakey [14]. MTX-Sepharose was obtained as described [15]. Complex bacteriological media were from Difco laboratories, and all media were prepared as described [16]. [^{35}S]dATP-$^{\alpha}$S triethylammonium salt (400 Ci/mmol) for DNA sequencing was supplied by Amersham International. Restriction endonucleases, Taq polymerase, T4 DNA ligase and restriction enzymes were purchased from Boehringer Mannheim Biochemicals. The TS and DHFR deficient *E. coli* PA414 was a gift

from Drs Ahrweiler and Frieden (University Medical School, St. Louis). Oligonucleotides were synthesized by the Analytical Services at the Instituto de Parasitología y Biomedicina, Granada.

2.2. DNA Manipulations

General methods for DNA manipulations were as described [16]. DNA fragments were purified by agarose gel electrophoresis using Geneclean® kit (BIO101).

2.3. Expression system construction

The coding sequence for the DHFR domain was amplified from the plasmid pTC4.5 which contains a 4.5-kb fragment of *T. cruzi* genomic DNA with the complete DHFR-TS sequence. We used the oligonucleotides DHFRstart and DHFRstop as terminal primers. DHFRstart, 5′-TTGAATTCATGTCGCTGTTTAA-3′, primes on the coding strand and contains a start codon (bold) and a *Eco*RI site (underlined). DHFRstop, 5′-GTCTGCAGCTAGTTGCGTGG-3′, primes on the non-coding strand and carries a *Pst*I site (underlined) and an engineered stop codon (bold). The polymerase chain reaction (PCR) was carried out under mineral oil in a volume of 50 μl. The reaction buffer contained 400 μM dNTPs, 2 mM $MgCl_2$, 25 pmol of each sense and antisense primers, 250 ng of plasmid DNA and 2.5 μl of Taq polymerase. PCR parameters were: 5 min at 95°C (1 cycle); 1 min at 95°C, 1 min 30 s at 45°C and a 2 min at 72°C (35 cycles); 10 min at 72°C, 1 cycle. After digestion of the purified PCR product with *Eco*RI and *Pst*I, it was cloned in pKK223.3 (Pharmacia). The expression construct (pKTCD) was propagated in XL1-Blue cells (Stratagene). Double strand DNA was sequenced by the dideoxy method [17], using the Sequenase Version 2.0 from U.S. Biochemical Corp. Reagents. pKTCD was used to transform the *E. coli* strains JM105 (lacI^q) (Pharmacia) and PA414.

2.4. Purification of the DHFR domain

Bacterial clones were grown at 37°C in LB [16] containing 100 μg ml^{-1} of ampicillin. The lacI^q cells were grown at 37°C to an A_{600} of 0.5–0.7, and protein expression was then induced with 1 mM isopropyl thiogalactoside and grown for a further 3–6 h. Recombinant DHFR domain was purified by methotrexate-Sepharose affinity chromatography as described for the bifunctional enzymes of *L. major* [6,18] and *T. cruzi* [13], followed by anion-exchange chromatography in a Mono Q HR5/5 column from Pharmacia as reported for the bifunctional enzyme of *Plasmodium falciparum* [19]. Bifunctional DHFR-TS from *T. cruzi* was obtained as described [13].

2.5. Protein analysis

Protein determinations [20], electrophoresis on SDS-PAGE and staining with Coomassie Blue R250 [21] were performed as described. The relative molecular mass of the DHFR domain in the native state was determined by gel filtration using a Superose-12 HR 10/30 column from Pharmacia. The equilibration and chromatography was performed in potassium phosphate 50 mM, pH 7.0; NaCl 0.15 M, at flow rate 0.5 ml min^{-1}. The column was calibrated with a mix of proteins of molecular masses ranging from 13.7 to 67 kDa purchased from Pharmacia.

2.6. Enzyme assays

DHFR activity determinations were performed spectrophotometrically by monitoring the decrease in absorbance at 340 nm at 25°C [22]. The standard assay contained 100 μM H_2folate, 100 μM NADPH, 50 mM Tes, pH 7.0, and 75 mM 2-mercaptoethanol. The reactions were initiated by addition of about 10 units of enzyme. One unit of enzyme activity is the amount of enzyme required to produce 1 nmol min^{-1} of product at 25°C using ϵ of 12 300 M^{-1} cm^{-1} at 340 nm.

2.7. Determination of kinetic parameters

K_m values for NADPH and H_2folate were obtained from kinetic assays by varying the substrate at subsaturating concentrations and fixing the other substrate at saturating concentrations. Non-linear least squares fit of the data to the

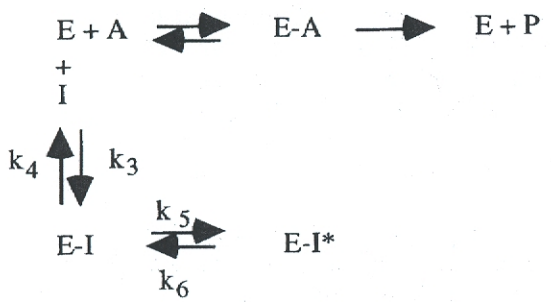
Michaelis-Menten equation was used to determine the K_m values and the k_{cat}.

2.8. Determination of TMP and Pyr K_i values

TMP and Pyr K_i values were obtained from the expression $I_{50} = K_i(1 + [S]/K_m)$ that, for competitive inhibitors, relates the concentration of inhibitor which inhibits DHFR activity by 50% (I_{50}) with the K_i value. K_m is the Michaelis-Menten constant for H_2folate and [S] is the concentration of H_2folate. The reactions were initiated with 2.5 and 0.3 nM of DHFR domain and bifunctional enzyme, respectively, and the concentration of H_2folate was 15 μM. Determinations for each inhibitor concentration were performed in duplicate.

2.9. Analysis of MTX inhibition

MTX inhibition was analyzed in experiments where both substrates, NADPH and H_2folate, were kept at saturating concentrations and the concentration of MTX was varied between 0 and 20 nM. The reactions were initiated with 0.85 nM of protein in the case of the bifunctional enzyme and 2.4 nM of protein, in the case of the DHFR domain. The onset of MTX inhibition was analyzed as described [23] for slow-binding inhibitors according to the following kinetic scheme (Scheme 1), where E represents the DHFR domain or the bifunctional enzyme, A represents H_2folate and I represents MTX. Progress curves reflect the slow establishment of the E-I ⇄ E-I*



Scheme 1.

equilibrium and were fitted to the general integrated Eq. (1) by non-linear regression,

$$A_{340} = v_f t - (v_f - v_i)(1 - e - kt)/k \quad (1)$$

introducing the absorbance and the time as variables. The equation reflects a pseudo-first order process, where v_i and v_f are the initial and final DHFR steady-state rates, k is the pseudo-first order constant and the t is the time. K_i of the initial inhibitory complex and k_5 were obtained employing the equation

$$1/k - k_6 = 1/k_5 + \frac{K_i(1 + [H_2\text{folate}]/K_m)}{K_5[\text{MTX}]} \quad (2)$$

where K_m is the Michaelis-Menten constant for H_2folate, and the k_6 value was obtained from each progress curve from the relationships $k_6 = kv_f/v_i$. The inhibition constant for the overall process, K_i^*, was calculated from $K_i^* = K_i k_6/(k_5 + k_6)$.

2.10. Determination of the apparent rate of dissociation of MTX

The rate of MTX dissociation was determined by a competitive method incubating the preformed radioactive ternary complex [^{3}H]MTX-NADPH-enzyme with an excess of cold MTX. The radioactive complex was obtained by incubating the protein with 100 μM NADPH and [^{3}H]MTX (37 Ci mmol^{-1}; NEN Research Products) in 1.2 ml of 50 mM Tes (pH 7.4), 2 mM DTT, and 1 mM EDTA, at 25°C, for 45 min (in the case of the DHFR domain the buffer contained also 8% polyethylene glycol 3350). The concentration of radioactive MTX was 0.1 μM for the bifunctional enzyme and 0.9 μM for the DHFR domain. The dissociation was initiated by addition of 100 μM of cold MTX and the release of the [^{3}H]MTX was followed separating the macromolecular-bound from free [^{3}H]MTX by filtering 100-μl aliquots of the reaction mix on small columns of Sephadex G-15 by a slight modification of a previously described method [4] and measuring the radioactivity of the complex isolated. The protein concentration in the experiments was 16.9 nM for the bifunctional enzyme and 200 nM for the monofunctional domain.

Table 1
Purification of the *T. cruzi* DHFR domain

Purification step	Total protein[a] (mg)	Sp. act. (U mg^{-1})	Total units	Purif. (*x*-fold)	yield[b] (%)
Crude extract	1218	42	51 200		
MTX-Sepharose	0.8	17 000	14 000	405	27.3
Mono Q	0.17	37 700	6500	900	13

[a]From 2 l culture.[b]Based on DHFR activity.

3. Results

3.1. Gene cloning and expression

Based on homology comparisons and secondary structure predictions with the previously reported DHFR-TS of *T. cruzi* [13], we considered up to residue Asn234 as the DHFR domain of the bifunctional enzyme. By means of PCR we amplified the considered domain introducing a stop codon after Asn234. The PCR product yields a single product of approximately 700 bp, which was purified from agarose, digested with *Eco*RI and *Pst*I, and cloned in pKK223.3 to give pKTCD. pKTCD was initially propagated in XL1-Blue cells and the authenticity of the construct was confirmed by restriction analysis and sequencing. The plasmid was used to transform several *E. coli* strains which were grown under suitable conditions of expression. Enzyme activity determinations revealed the expression of a catalytically active DHFR in every *E. coli* strain checked. However, the expression level was somewhat low (0.1–0.2%) and the protein was undetectable by SDS-PAGE stained with Coomassie blue.

3.2. Purification and characterization of the T. cruzi DHFR domain

The DHFR domain of *T. cruzi* was purified from PA414 *E. coli* cells to homogeneity. Purification was performed by MTX-Sepharose affinity using the same conditions as those described for the *T. cruzi* bifunctional enzyme [13], although an additional anion-exchange chromatography step in a mono Q column was necessary (Table 1). The purified product displayed a single 27-kDa band on SDS-PAGE (Fig. 1), which is in agreement with the size expected from the amino acid sequence (25 768 Da). The molecular mass of the native DHFR domain obtained by gel filtration was approximately 20 kDa, which indicated that it behaved as a monomer. The purified protein was highly stable, and there was no loss of activity after long incubations at room temperature and after several cycles of freezing-unfreezing. However, the protein underwent a progressive loss of activity when it was diluted at concentrations below 6 $\mu g\ ml^{-1}$. This effect was prevented by adding polyethylene glycol at 8% in the elution buffer. The specific activity of the purified DHFR domain was 38 000 U mg^{-1}, which is similar to the specific activity obtained for the bifunctional enzyme.

The K_m values for NADPH and H_2folate were 16 ± 1 and 0.75 ± 0.06 μM, respectively. Taking into consideration that the domain exists as a monomer and using the value of 25 768 Da as the molecular mass of the enzyme, we obtained a k_{cat} value of $16.5 \pm 0.5\ s^{-1}$. Table 2 summarizes the kinetic parameters of the DHFR domain compared to those of the bifunctional protein [13].

We have compared the effects of pH and concentration of KCl on *T. cruzi* DHFR-TS and the DHFR domain (Fig. 2). Both proteins displayed a pH-independent activity between pH 6.1 and 7.2, while the activity declined at basic pH, with half maximal activity around pH 8. KCl activated the DHFRs from both sources approximately 40%, in the range of salt concentrations spanning between 0.05 and 0.25 M. Both proteins were very stable at high KCl concentrations and at 1 M the DHFR domain and the bifunctional enzyme conserved 55 and 36% activity, respectively.

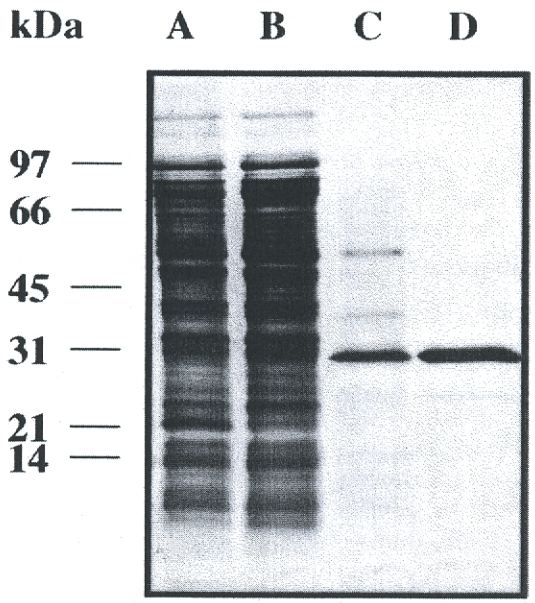


Fig. 1. Purification of the recombinant DHFR domain: 15% SDS-PAGE stained with Coomasie R-250. Lane A, 20 μg crude soluble extract; Lane B, flow through from MTX-Sepharose column; Lane C, 2 μg after MTX-Sepharose chromatography; lane D, 2 μg after anion-exchange chromatography in a mono Q column.

3.3. Inhibition by TMP and Pyr

TMP and Pyr were tested as antifolate inhibitors of DHFR activity. The progress of the reaction versus the time at different concentrations of the inhibitors displayed straight lines for both, the *T. cruzi* DHFR domain and wild-type bifunctional enzyme, which confirmed the competitive character of TMP and Pyr (data not shown). K_i values and I_{50} data for the related inhibitors are shown in Table 3.

3.4. Analysis of the interaction with MTX

The inhibition of both proteins in the presence of MTX led to two-asymptotic progress curves of the reaction versus time (Fig. 3), indicating the slow-binding character of MTX [23]. The form of the progress curves could be interpreted assuming a two-step inhibition process (Scheme 1, Table 4), where the initial step involves a rapid formation of a initial enzyme-inhibitor complex that subsequently undergoes a slow isomerization. From analysis of the progress curves we determined that the overall MTX inhibition constant, K_i^*, for the *T. cruzi* DHFR domain was 9.2 pM, 2-fold higher than for the wild-type bifunctional enzyme, which was 4.9 pM. The inhibition constant for the initial inhibitory complex, K_i, was also approximately 2-fold increased (K_i 400 pM) compared to the bifunctional enzyme ($K_i = 230$ pM). The low values of K_i for both proteins indicated that binding is tight in the initial complex. However, the subsequent isomerization is also significant and increases the tightness of MTX binding to both proteins about 45-fold. The values obtained for the rate constants of direct, k_5, and reversal, k_6, isomerization were 2.3 min^{-1} and 0.051 min^{-1} for the wild-type enzyme and 4.7 and 0.11 min^{-1} for the DHFR domain, respectively. The ratio k_5/k_6 governs the isomerization process and in the present case, where $k_5 \gg k_6$, its value agrees with the ratio K_i/K_i^*, for both enzymes. The rate of MTX release from the ternary complex is limited by the rate of reversal isomerization and the effective off-rate is indicated by the apparent rate constant of dissociation, $k_{off,app}$. The dissociation of radioactive MTX from the ternary complex

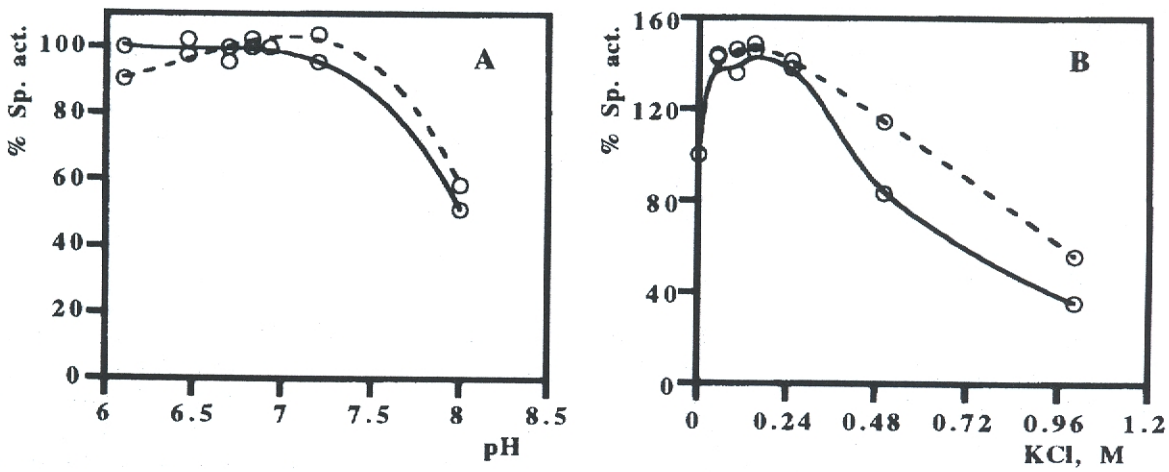


Fig. 2. Effects of pH and KCl on the activity of DHFR-TS from *T. cruzi* and the DHFR domain. Initial activities were determined in standard reaction buffers prepared at pH 6.1, 6.5, 6.7, 6.8, 6.9, 7.2 and 8, and expressed in relation to the maximum activity obtained at pH 7.0. For the assay of the effects of KCl, initial activities were determined in standard buffers prepared at the concentrations of desired salt, and expressed in relation to the standard buffer without KCl. The solid line represents the activity of DHFR-TS from *T. cruzi* and the dashed line represents the activity of the DHFR domain. (A) Effect of pH. (B) Effect of KCl.

formed with NADPH and both, the DHFR domain and *T. cruzi* DHFR-TS, appeared to consist of a single first-order process, with $k_{off,app}$ values of 0.034 min^{-1} for the wild-type enzyme and 0.040 min^{-1} for the DHFR domain, which corresponded with a $t_{1/2}$ of 20 and 17 min, respectively. From the [^{3}H]MTX dissociation experiments we determined that 0.8 mol of MTX were bound per mol of dimeric bifunctional enzyme indicating that the stochiometry of MTX binding was 1:1, which agrees with the observation made using the *L. major* [6] and *Crithidia fasciculata* [3] enzymes. The DHFR domain also bound 1 mol of MTX per mol of monomeric protein indicating that the stochiometry is 1:1 and that the domain is 100% active.

4. Discussion

DHFR-TS from *T. cruzi* is a bifunctional enzyme in which the two catalytic activities are located on the same polypeptide chain. The DHFR domain is at the amino terminus. Although it is the more variable domain, it conserves high similarities with other DHFRs, especially with regard to the residues assigned to NADPH and H_2folate binding [24–26]. In contrast, the homology at the carboxy terminus of the DHFR domain, is not well defined [13] and the algorithms of secondary structure prediction [27,28] do not indicate the β-sheets, β-G and β-H, which are observed in all the DHFRs of known tertiary structure. However, secondary structure analysis for the complete *T. cruzi* DHFR-TS predicts an α-helix spanning from Arg235 to Glu250, which is present at the solved structures of the *Lactobacillus casei* and *E. coli* TSs [29,30], and in addition, significant homology between the DHFR-TS of *T. cruzi* and other TSs begins at Arg235 [13]. Therefore, we chose Asn234 as the last amino acid belonging to the DHFR domain and expressed it under the tac promoter in the expression vector pKK223.3. Asn234 aligns with Asn231 in *Plasmodium falciparum* which has also been successfully selected as the last amino acid in the expression of the DHFR domain [31].

The catalytically active DHFR domain was purified to homogeneity by means of MTX affinity chromatography followed by anion-exchange chromatography in a mono Q column. Several properties were similar to those of the wild-type bifunctional enzyme; it was very stable

Table 2
Kinetic parameters of the DHFR domain and the *T. cruzi* bifunctional enzyme

Substrate	DHFR domain		Bifunctional DHFR-TS[a]	
	K_m (μM)	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})[b]
H_2folate	0.75 ± 0.06	16.7 ± 0.2	1.2 ± 0.1	36 ± 4
NADPH	16 ± 1	16.2 ± 0.5	17 ± 2	36 ± 4

[a]From Reche et al. [13].[b]Per monomer of enzyme.

and presented a specific activity of around 37 000 U mg^{-1}. In contrast, the native *T. cruzi* DHFR domain behaved as a monomer of 20 kDa upon gel filtration, which suggested that if direct contacts exist between the DHFR domains in the dimer, they must be weak and assisted by others much stronger belonging to the TS domains. Both the DHFR domain and the wild-type enzyme showed similar kinetic properties. KCl activated both enzymes and the activity was pH-independent from pH 6 to 7.0, declining rapidly when the pH increased. The effect of pH on DHFR activity resembled that described for the bacterial enzymes [32–34]. In contrast, DHFR from vertebrates are pH independent from pH 6 to 9 [35–37]. The K_m values determined for NADPH and H_2folate were in good agreement with those obtained previously for the bifunctional enzyme [13] although the k_{cat} for the *T. cruzi* DHFR domain was 16.5 s^{-1}, while the k_{cat} for the bifunctional enzyme was 36 s^{-1} (per monomer). These small differences observed between the kinetic parameters, could be due to the cancellation of interactions that existed in the bifunctional enzyme. However, these interactions are not required for catalysis and the *T. cruzi* DHFR domain is practically as efficient as the wild-type enzyme.

DHFR-TS from *T. cruzi* is not potently inhibited by the classical anti-microbial antifolates TMP and Pyr, which were in addition poorer inhibitors of the DHFR domain compared to wild-type DHFR-TS. This low sensitivity of DHFR to TMP and Pyr has been previously reported in assays performed with *T. cruzi* crude extracts [38]. The sensitivity of *T. cruzi* DHFR to these analogues was increased with regard to *L. major* DHFR [39], and is more similar to the human enzyme (K_iTMP = 110 nM; K_iPyr = 65 nM) than to most microbial enzymes. For example the K_iPyr (employed as an anti-malarial) for DHFR-TS from *P. falciparum* is 0.1 nM [19] and the K_iTMP (employed as a bactericidal) for the DHFR from *E. coli* is 0.08 nM [40]. This low sensitivity of *T. cruzi* DHFR to TMP and Pyr could be one of the possible reasons why they have not been useful in the treatment of Chagas' disease.

MTX was a potent inhibitor of both the DHFR domain and the wild-type bifunctional enzyme from *T. cruzi*. As in the case of bacterial DHFRs [41,42,11] and vertebrate DHFRs such as chicken [40,41] and human [12], the pattern of MTX inhibition can be interpreted in terms of an initial step of binding of the inhibitor followed by an isomerization of the initial complex formed. In the case of the DHFR domain and DHFR-TS from *T. cruzi*, this potential conformational change contributed in the same measure to the overall binding of MTX, increasing tightness of binding by a factor of about 45. However, the initial step of MTX binding is slightly modified in the case of the *T. cruzi* DHFR domain and the inhibition constant, K_i, is approximately 2-fold increased, being the reason why the overall inhibition constant of MTX, K_i^*, is also elevated 2-fold. On the other hand, the K_i of the initial step of MTX-binding is comparable to the one-step binding of competitive inhibitors such as TMP and Pyr, whose K_i values were also increased compared to those of the *T. cruzi* bifunctional enzyme. The inhibition by MTX of *T. cruzi* DHFR is more similar to the human enzyme than to *E. coli* DHFR [12]. Thus, in the case of the human enzyme, the K_i of the initial inhibitory complex is

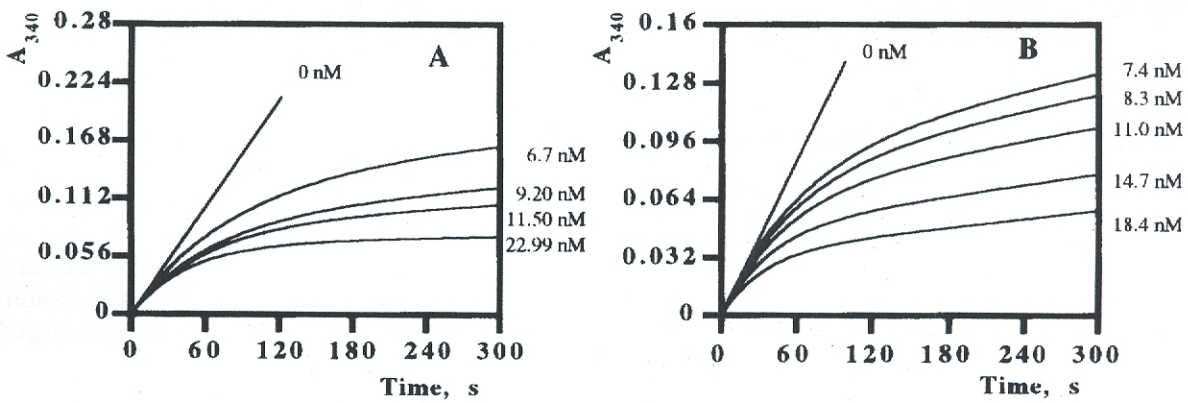


Fig. 3. MTX inhibition of DHFR activity. (A) Progress curves for MTX inhibition of DHFR-TS of *T. cruzi*. Reactions were initiated with 0.85 nM of enzyme. (B) Progress curves for the DHFR domain of *T. cruzi*. Reactions were initiated with 2.4 nM of enzyme. The concentration of MTX is indicated for each curve.

200 nM and the isomerization process increases the overall binding 60-fold. In contrast, for *E. coli* DHFR, the K_i of the initial inhibitory complex is 2.4 pM and the contribution of the conformational change to overall binding of MTX is only 3-fold [11].

There is experimental evidence showing the existence of DHFR interactions in *L. major* DHFR-TS. However it is unclear if such interactions occur directly, via DHFR-DHFR contacts, or indirectly, through the TS domains. The present results, and previous reports with the bifunctional enzymes of *Crithidia fasciculata* [3] and *L. major* [6] showed that, despite the fact that the enzyme has two folate binding sites, only one can bind MTX. Hence, functional DHFR-DHFR domain communication should exist, as binding of MTX to one domain prevents binding to the other. In contrast, this communication disappears in the *T. cruzi* DHFR domain, which behaves as a monomer upon gel filtration and where every folate binding site can bind MTX. Moreover, the X-ray structure of *L. major* DHFR-TS [7] does not evidence physical DHFR-DHFR interactions and, surprisingly, the two folate binding sites of each DHFR domain are occupied by MTX. However, the crystals were obtained under conditions in which the TS sites were taken up by inhibitors (FdUMP and 10-propargyl-5,8-dideazafolate) and it is possible that under this situation, DHFR-DHFR contacts have been canceled. Indeed, it has been observed that partial proteolysis of the TS domain of *L. major* DHFR-TS produced TS inactivation and a loss of DHFR-DHFR interactions, reflected by the fact that each DHFR domain could bind one mol of MTX [43]. If physical DHFR-DHFR interactions are weak and assisted by stronger ones between the TS domains, the perturbation or inhibition of the TS domain could modify those interactions. Hence, it is possible that contacts between DHFR domains exist in the bifunctional enzyme although a crystal structure of the free enzyme would be required to evidence them.

Table 3
Inhibition constants of TMP and Pyr for the DHFR domain and the *T. cruzi* bifunctional enzyme

	DHFR-TS		DHFR domain	
	K_i (μM)	I_{50} (μM)	K_i (μM)	I_{50} (μM)
Trimethoprim	0.024	0.36[a]	0.150	3.0[c]
Pyrimethamine	0.012	0.19[b]	0.083	1.5[d]

The H_2folate concentrations were: [a]16.5, [b]18, [c]14.08 and [d]12.6 μM.

In summary, we have expressed the DHFR domain of *T. cruzi* DHFR-TS as a catalytically active protein. The purified *T. cruzi* DHFR domain has similar properties and similar kinetic parameters to the wild-type enzyme. Kinetic ex-

Table 4
Parameters of MTX inhibition for *T. cruzi* bifunctional DHFR-TS and the DHFR domain

	k_5 (min^{-1})	k_6 (min^{-1})	k_5/k_6	K_i (pM)[a]	K_i^{*}[b] (pM)
DHFR-TS	2.3	0.051	45	230	4.9
DHFR domain	4.7	0.11	43	400	9.2

k_5 and k_6 values were obtained from the progress curves. The K_m values for H_2folate were 1.2 and 0.75 μM for the *T. cruzi* bifunctional enzyme and the DHFR domain, respectively. The H_2folate concentration used in these assays was 62.5 μM.[a]K_i for the initial inhibitory complex.[b]K_i overall.

periments with TS coupled to the DHFR domain are now possible and obtaining a structure may be easier than for the bifunctional protein. The availability of the DHFR domain from *T. cruzi* DHFR-TS could help in the understanding of special features of the bifunctional enzyme and facilitate the rational design of specific drugs for treatment of Chagas' disease.

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